Chapter III

A Simple Microbiological Method for Determining Presence and for Classifying of Antibiotic Residues in Marine Food Products

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A Simple Microbiological Method for Determining Presence and for Classifying of Antibiotic Residues in Fish Products

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SIMPLE EXAMINATION METHOD FOR ANTIBIOTIC RESIDUES IN MARINE FOOD PRODUCTS

Drug residue in aquacultured shrimp present concerns to health authorities and consumers. The best measure to prevent a health threat to consumers is the proper management of farm, and if drugs are needed, they are to be used with care, correct amount and type. In circumstances where control is in the hand of processors, determination of drug residue in raw material fifteen to twenty one days before harvesting for residue level in raw material is recommended. Detection of residue when the raw material is received at the factory is only good for establishing a suppler history.

Drug residues can be detected by either a simple microbiological assay or sophisticated chemical methods.

The following method is a simple microbiological assay developed by the Ministry of Health and Welfare, Japan for testing a few groups of antibiotics, and could be utilised by processors and regulators for screening purposes.

1. Materials

I) Assay Strain
- a) *Micrococcus luteus* ATCC 9341 (hereinafter called *M. luteus*)
- b) *Bacillus subtilis* ATCC 6633 (hereinafter called *B. subtilis*)
- c) *Bacillus mycoides* ATCC 11778 (hereinafter called *B.mycoides)*

II) Culture Medium
- a) Agar medium for storage and maintenance Ordinary agar medium (Nutrient Agar)*[1)]
- b) Liquid medium for propagation Bouillon for measurement of sensitivity (Sensitive Test Broth)*[1)]
- c) Medium for mixing with assay strain
  - (1) Antibiotic Medium 5 (Difco) (hereinafter called AM5)
  - (2) Antibiotic Medium 8 (Difco) (hereinafter called AM8)

*[1)] Nissui Seiyaku's products or similar mediums.

III) Petri Dish
Sterilised Petri dish with an inner diameter of 86±1mm.

IV) Paper Disk

Paper disks with a diameter of 10mm and thickness of 1.1-1.2 mm are used*[2].

The paper disks are sterilized for 15 min at 121°C and are completely dried before use.

*[2] Toyo Roshi's (filter) products for Antibiotics assay paper disks of meat or a paper disk of required thickness cut to size.

V) Buffer Solution

a) *Citric Acid Acetone Buffer Solution*

Prepare the buffer solution by mixing of 30vol.% distilled water, 35vol.% acetone, and 35vol.% mixture of 0.2M citric acid solution and 0.5M potassium hydroxide solution in the same volume.

Prepare the 0.2M citric acid solution with 4.2g of monohydrate citric acid ($C_6H_8O_7$:MW210.14) which is dissolved in distilled water with a total volume of 100ml.

Prepare the 0.5M potassium hydroxide solution with 2.8g potassium hydroxide (KOH:MW56.11) which is dissolved in distilled water with a total volume of 100ml.

b) *pH 4.5 Phosphate Buffer Solution*

Prepare the pH4.5 buffer solution with 13.6g of potassium dihydrogen phosphate (KH_2PO_4:MW136.09) which is dissolved in distilled water with a total volume of 1000ml.

c) *pH 6.0 Phosphate Buffer Solution*

Prepare the pH6.0 buffer solution with 8.0g potassium dihydrogen phosphate (KH_2PO_4: MW136.09) and 2.0g dipotassium hydrogenphosphate (K_2HPO_4:MW174.18) which are dissolved in distilled water with a total volume of 1000ml.

d) *pH 8.0 Phosphate Buffer Solution*

Prepare the pH 8.0 buffer solution with 0.523g potassium dihydrogen phosphate (KH_2PO_4:MW136.09) and 16.73g dipotassium hydrogenphosphate (K_2HPO_4:MW174.18) which are dissolved in distilled water with a total volume of 1000ml.

Note: The phosphate buffer solutions of pH 4.5, 6.0 and 8.0 are preferred to be prepared just before they are used. The solutions should be stored under sealed condition after being sterilised for 15 min at 121°C under high pressure. Solutions with precipitates and turbidity can not be used.

2. Storage and Maintenance of Assay Strain

I) *M. luteus*

a) Incubate the assay strain on a slant nutrient agar at 30°C for 18 hours. After being confirmed that *M. luteus* spreads on the medium, then seal it with a sterilized rubber stopper and store in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Seed purely cultured thick fresh bacteria (multiplied on nutrient agar medium plate) into 1 ml. nutrient broth with 10-20% defated (skim) milk or with glycerin and seal it with a sterilized rubber stopper and store it in a freezer*[3]. It can be kept (stored) for longer duration than a slant nutrient agar medium.

c) Keep the assay strain in a refrigerator or freezer using freeze-drying method.

*[3] The concentration of glycerin is 10-16% when it is preserved at -70°C, 40% when at -20°C.

II) *B. subtilis*

a) Incubate the assay strain on a slant nutrient agar medium at 30°C for 18 hours. After being confirmed that *B. subtilis* spreads on the medium, seal it with a sterilized rubber stopper and store in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Subdivide the assay strain which is produced by (3. Preparation of Assay Strain Solution); those for short storage within one month are put in a refrigerator, those for longer storage are put in a freezer.

c) Keep the assay strain in a refrigerator or a freezer using freeze-drying method.

III) *B. mycoides*

a) The assay strain is incubated on a slant nutrient agar medium at 30°C for 18 hours. After being confirmed that *B. mycoides* spreads on the medium, it is then sealed with a sterilized rubber stopper and stored in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Subdivide the assay strain which is produced by (3. Preparation of Assay Strain Solution); those for short storage within on month are put in a refrigerator, those for longer storage are put in a freezer.

c) Keep the assay strain in a refrigerator or a freezer using freeze-drying method.

Note: It's convenient when these assay strains are maintained and stored in several different methods. Each method mentioned above can be used for the maintenance. The best way is using all the 3 methods to maintain the assay strains.

3. Preparation of Assay Strain Solution

I) *M. luteus*

Inoculate the stored assay strain into the Sensitivity test broth, incubate it for 18 hours at 30°C , maintain three generations, use the medium solution of the third generation as assay strains.

II) *B. subtilis*

Apply the stored assay strain on a nutrient agar medium plate, culture it for one week at 30°C to produce spores*[4]. Scrape the grown bacteria on the plate, put it into sterilized physiological saline solution and heat it up for 30 min at 65°C. Then centrifuge it for 20 min at 3000rpm, throw away the supernatant. Put the sediment into sterilized physiological saline solution again, and use it as the spore solution.

Produce serial dilutions of the spore solution, mix a 1% dilution into AM5 which is kept at 50°C flow 8ml of the mixture into a petri dish to form a plate. Put a paper disk wetted with 0.5μg/ml kanamycin solution on each plate and culture them for 18 hours at 30°C . Find out the concentration whose inhibition zone is 14±1 mm in diameter. Produce the dilution with the same concentration as the assay strain solution. The number of spores in the assay strain solution is about 10^7-10^8/ml.

III) *B. mycoides*

Apply the stored assay strain on a nutrient agar medium plate , culture it for one week at 30°C to produce spores*[4]. Scrape the grown bacteria on the plate, put it into sterilized physiological saline solution and heat it up for 30 min at 65°C. Then centrifuge it for 20 min at 3000rpm, throw away the supernatant. Put the sediment into sterilized physiological saline solution again, and use it as the spore solution.

*[4] Dye the spores, see them under a microscope and confirm that there are 80% spores in a visual field of microscope. If the productivity of spores is not enough, incubate the plate for few more days to multiple spores. If even after an incubation period of more than 10 days, the number of spores is still not enough, it is considered that there are something wrong with the assay strain and do not use such assay strain.

Produce dilutions with gradual decreasing method from the spore solution, mix a 1% dilution into AM8 which is kept at 50°C, flow 8ml of the mixture into a petri dish and to form a plate. Put a paper disk wetted with 0.25μg/ml oxytetracycline

solution on each plate and culture them for 18 hours at 30°C. Find out the concentration whose inhibition zone is 14±1mm in diameter. Produce the dilution with the same concentration as the assay strain solution. The number of spores in the assay strain solution is about 10^7-10^8/ml.

4. Preparation of Assay Plates

I) *M. luteus* Plate

Add *M. luteus* assay strain solution into five times AM5 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.025μg/ml ampicillin solution on the plate, incubate it for 18 hours at 30°C, the diameter of inhibition zone must be 14±1mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

II) *B. subtilis* Plate

Add *B. subtilis* assay strain solution into one hundred times AM5 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.5μg/ml kanamycin solution on the plate, and incubate it for 18±1 hours at 30°C, that the diameter of inhibition zone must be 14±1mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

III) *B. mycoides* plate

Add *B. mycoides* assay strain solution into one hundred times AM8 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each Petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.25μg/ml oxytetracycline solution on the plate, culture it for 18±1 hours at 30°C, the diameter of inhibition zone must be 14±1 mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

*[5] When the following reasons: (1) a variation in assay strain, (2) a mistake in amount of bacteria to be inoculated, (3) a mistake in preparation of assay medium plates, and (4) wrong

concentrations of antibiotic in paper disks, etc., are considered, the preparation should be carried out again.

Note: The inhibition zones which form in the plates of *M. luteus*, *B. subtilis* and *B. mycoides* should have a clear boundary and has no colony inside the zone and the assay strain on the plate must be smooth without spots. The plates which do not meet the above conditions cannot be used since they may have a variation and may be contaminated.

5. Standard Solution of Antibiotics

Prepare a standard solution having a potential titre equivalent to 1000μg/ml of the standard antibiotics. Use antibiotics with a known factor (in the unit of 0.1mg) for this preparation. Using a sterilized messpipette, dilute the standard solution with the phosphate buffer solutions following minimum inhibition concentration method to prepare the working standard solution. The messpipette should be changed every time to dilute different solutions. Basically, the 1000μg/ml standard solution is prepared for every measurement.

The working solution can also be prepared using 10 times dilution method.

Example: Prepare standard solution of ampicillin.
If the potential on the label of bottle containing sodium ampicillin is 840g, weight exactly 10mg of the powder and dissolve it in 8.4ml sterilized distilled water, then a solution with titre of 1000μg/ml is formed.

Prepare the following working solution with the same method:

I) Ampicillin Standard Solution
Prepare the 1000μg/ml ampicillin standard solution by dissolving sodium ampicillin in sterile distilled water. Use pH6.0 phosphate buffer solution for dilution.

II) Kanamycin Standard Solution
Prepare the 1000μg/ml kanamycin standard solution by dissolving kanamycin sulfate in sterile distilled water. Use pH8.0 phosphate buffer solution for dilution.

III) Oxytetracycline Standard Solution
At first, dissolve oxytetracyclin hydrochloride in a small amount of 0.1N HCl and add necessary amount of sterile distilled water to prepare a standard solution of oxytetracycline of 1000μg/ml. Use pH4.5 phosphate buffer solution for dilution.

P.S. Dissolve macrolide antibiotics in a small amount of methanol first, then add sterilized distilled water to prepare standard of each macrolide antibiotic solution of 1000μg/ml . For dilution, use pH8.0 phosphate buffer solution.

6. Preparation of Sample Solution

Weigh 5g of muscles, meat or internal organs, add 20ml citric acid-acetone buffer solution, homogenize it, filter it with a filter paper, and the filtrate is the sample solution. If the filtering is difficult, centrifuge it for 15 min at 3000rpm, filter the supernatant.

7. Testing Method

Dip a paper disk into the sample solution, put the paper disk on a assay plate*[6], and press down gently with a pair of forceps. Keep it in a refrigerator for more than 30 min, then incubate it for 18 hours at 30°C.

Use the paper disk wetted with citric acid-acetone buffer solution as a negative control.

*[6] For one sample solution, use more than two paper disks for respective assay plates.

8. Judgement

When the diameter of inhibition zone is greater than 12mm, the result is positive*[7]. Confirm that the negative control of citric acid-acetone result is negative.

*[7] Clear inhibition zone shows that the result is positive and indicates the presence of inhibitory substances to the test organisms in the sample, otherwise it is negative.

CLASSIFYING AND ESTIMATING METHOD FOR ANTIBIOTIC RESIDUES IN MARINE FOOD PRODUCTS

1. Materials

I) Assay Strain
- a) *Micrococcus luteus* ATCC 9341 (hereinafter called *M. luteus*)
- b) *Bacillus subtilis* ATCC 6633 (hereinafter called *B. subtilis*)
- c) *Bacillus mycoides* ATCC 11778 (hereinafter called *B. mycoides*)

II) Culture Medium
- a) Agar medium for storage and maintenance
  Ordinary agar medium (Nutrient agar)*[8]
- b) Liquid medium for propagation
  Bouillon for measurement of sensitivity (Sensitivity test broth)*[8]
- c) Medium for mixing with assay strain
  - (1) Antibiotic Medium 5 (Difco) (hereinafter called AM5)
  - (2) Antibiotic Medium 8 (Difco) (hereinafter called AM8)

*8) Nissui Seiyaku's products or similar mediums.

III) Petri Dish

Sterilised petri dish with an inner diameter of 86±1mm.

IV) Paper Disk

Paper disks with a diameter of 10mm and thickness of 1.1-1.2mm are used*9).
The paper disks are sterilized for 15 min at 121°C and are completely dried before use.

*9) Toyo Roshi's (filter) products for Antibiotics assay paper disks of meat or a paper disk of required thickness cut to size.

V) Buffer Solution

a) *pH4.5 Phosphate Buffer Solution*
Same as mentioned in session {b) pH4.5 Phosphoric Acid Buffer Solution of V) Buffer Solution of (1. Materials)} in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

b) *pH8.0 Phosphate Buffer Solution*
Same as mentioned in the session {d) pH8.0 Phosphoric Acid Buffer Solution of V) Buffer Solution of (1. Materials)} in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

c) *pH4.0 Macllvaine Buffer Solution*
To prepare this buffer solution, mix 12.29ml of 0.1M citric acid solution and 7.71ml of 0.2M disodium hydrogenphosphate solution.

d) *pH4.0 Macllvaine Buffer Solution with Containing 0.01M EDTA-2Na*
Add ethylene diamine tetraacetate disodium salt (EDTA-2Na) in pH4.0 Macllvaine buffer solution to prepare its 0.01M solution.

e) *pH3.0 Macllvaine Buffer solution*
Prepare this buffer solution by mixing 15.86ml of 0.1M citric acid solution and 4.11ml of 0.2M disodium hydrogenphosphate solution.

VI) Column

a) SEP-PAK C18 Cartridge*10) (hereinafter called C18 Column)
C18 Column is treated with 5ml methanol, 5ml distilled water and 5ml saturated EDTA-2Na solution. The flow rate for the treatment is 1.5 ml/min.

b) Baker 10 CARTRIDGE carboxylic acid extraction column*11)
First pump 5ml of hexane into the COOH column and vacuate it for about 1 min. Then the column is treated with 5ml of methanol, 5ml of distilled water and 5ml of pH 4.0 Macllvaine buffer solution. The flow rate for the

treatment is about 1.5ml/min. The column is wetted by pH4.0 Macllvaine buffer solution until charge with the sample.

*10) The product of Waters (Millipore Co.) or the similar product.

*11) The product of J.T. Baker Inc. or the similar product.

2. Storage and Maintenance of Assay Strain

Same as mentioned in session (2.Storage and Maintenance of Assay Strain) in "Simple Examination Method For Antibiotic Residues in Livestock and Marine Food Products (Revised)".

3. Preparation of Assay Strain Solution

Same as mentioned in session (3. Preparation of Assay Strain Solution) in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

4. Preparation of Assay Plates

Same as mentioned in session (4. Preparation of Assay Plates) in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

5. Preparation of Sample Solution

Add a 10g sample in 30ml of pH4.0 Macllvaine buffer solution which contains 0.01M EDTA. Homogenize it and centrifuge it for 15 min at 3000rpm. Gather the supernatant, add 10ml hexane into it, mix them well by stirring, then centrifuge the mixture for 15 min at 3000rpm. Gather the aqueous layer, add 30ml chloroform into it, mix them well by stirring, then centrifuge the mixture for 15 min at 3000rpm. Gather chloroform layer, evaporate it to dryness under reduced pressure at less than 40°C. Dissolve the residue in 1ml of pH8.0 phosphate buffer solution. The resulted solution is the sample solution A.

On the other hand, flow the aqueous layer into the C18 column, then into the COOH Column. Wash the C18 column with 10ml distilled water, then flow with methanol. Throw the first 0.5ml water away and gather 5ml effluent (methanol). Evaporate it to dryness under reduced pressure at less than 40°C. Dissolve the residues into 1ml pH4.5 phosphoric acid buffer solution. The resulted solution is the sample solution B.

Pass pH3.0 Macllvaine buffer solution into the COOH Column, gather the 5ml effluent. Use 5N NaOH and 1N NaOH solutions to adjust the pH of the effluent to pH7.5. The effluent is the sample solution C.

6. Testing Method

Put the paper disks wetted with the samples solution on the assay plates of *B. subtilis, M. luteus* and *B. mycoides* and press down gently with a pair of forceps. Keep these

plates with the paper disks in a refrigerator for 30 min, then incubate them for 18 hours at 30°C.

7. Judgement

When the diameter of inhibition zone is greater than 12mm, the result is positive. Estimate the type of antibiotics remained in the livestock and marine food products from the sensitivity pattern of the three assay strains shown in *Table 1*.

Furthermore, penicillin is inactivated with penicillinase.

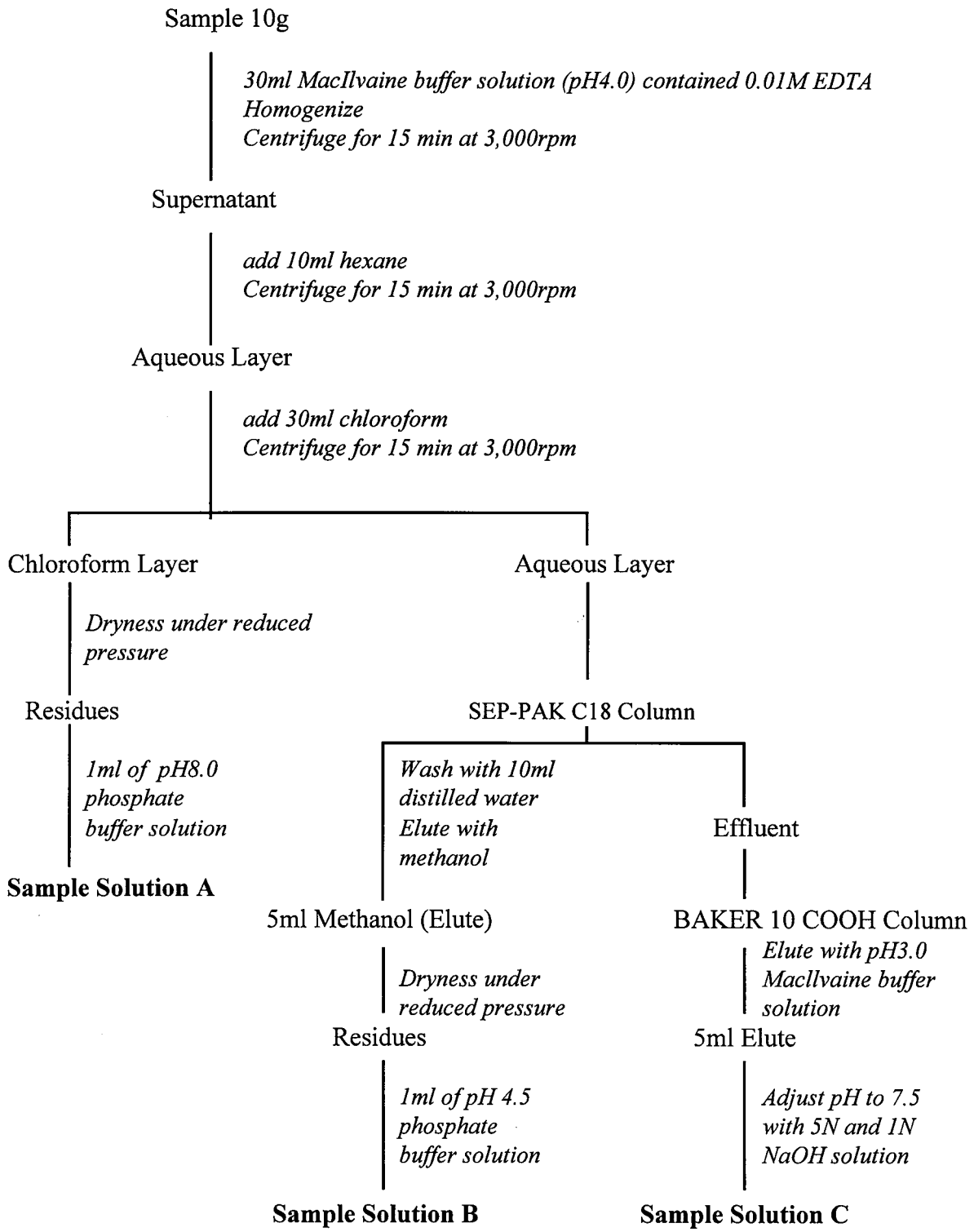
Note: When there are inhibition zones formed in all the three plates with, dilute the sample solution to a suitable concentration and test again.

Table 1: Classification and Estimation of Antibiotics by Sensitivity Pattern of Assay Strains

Sample Solution	Assay Strain			Antibiotics
	B. subtilis	*M. luteus*	*B. mycoides*	
A	+	++	-	Macrolides
	-	+	-	
B	+	-	++	Tetracyclines
	-	-	+	
	+	++	-	Penicillins
	-	+	-	
C	++	-	+	Aminoglycosides
	+	-	-	

Note:
++ represents bigger inhibition zone (diameter) than that shown by + .
- means no inhibition zone formed.

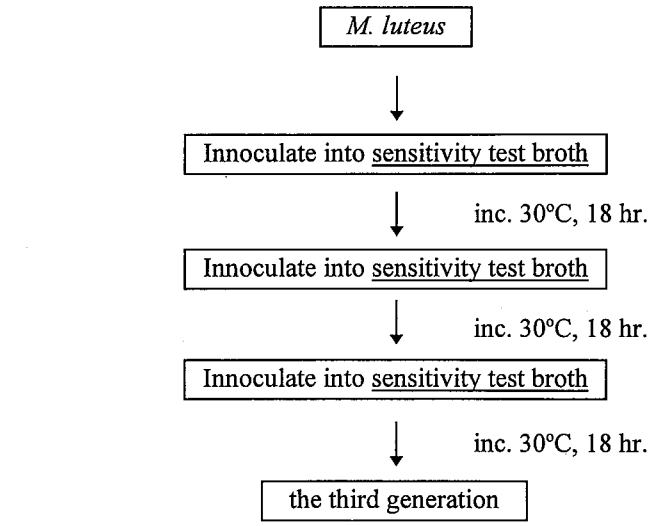
Figure1: Preparation Of Sample Solutions For Estimating Antibiotics in Livestock And Marine Food Products

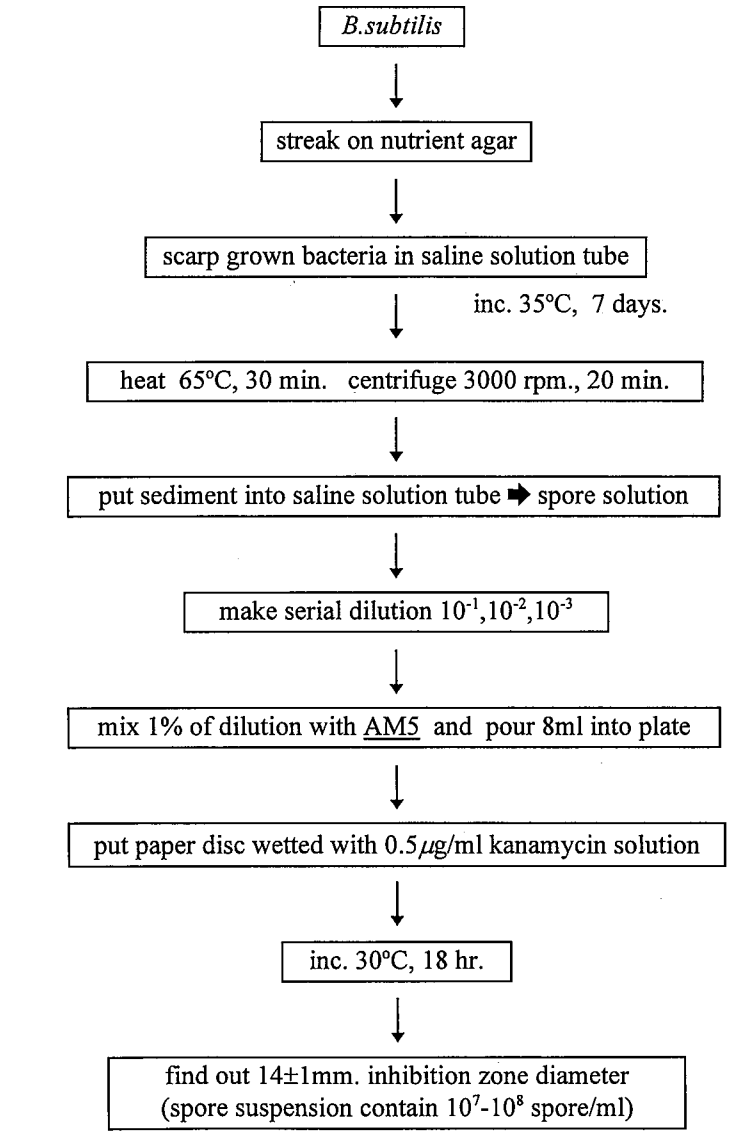


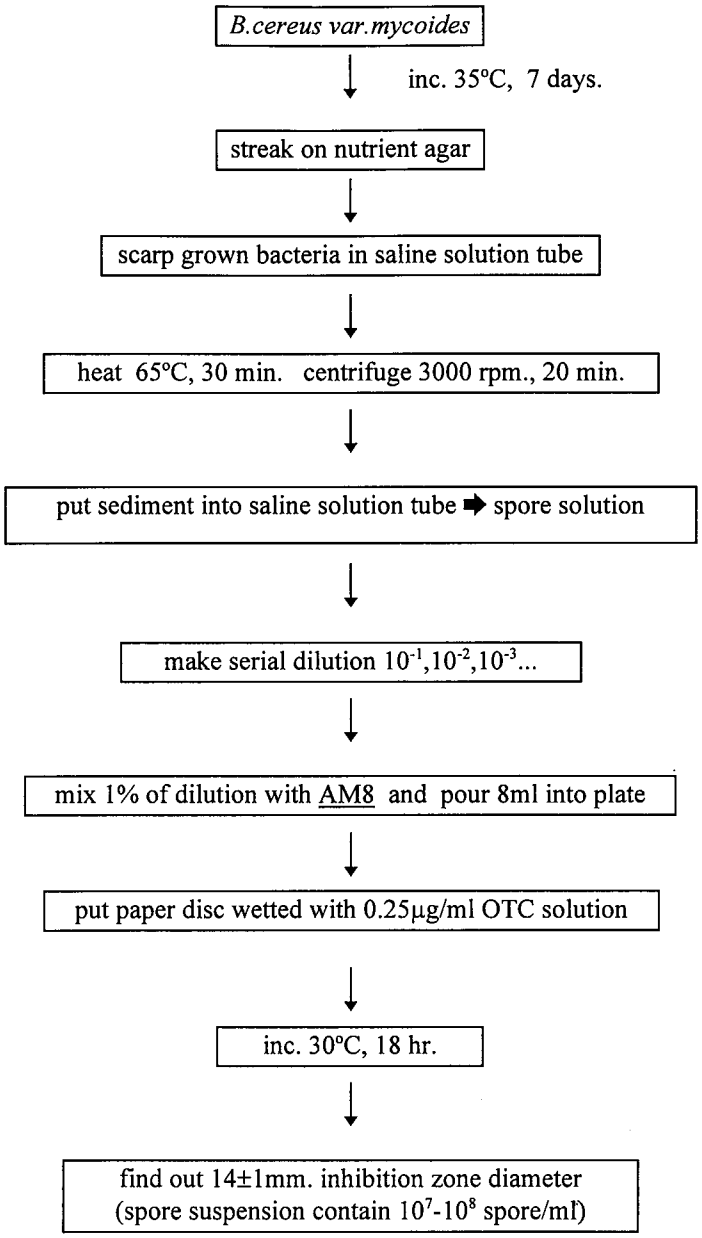
Reference:

Method developed by the Ministry of Health and Welfare, Japan.

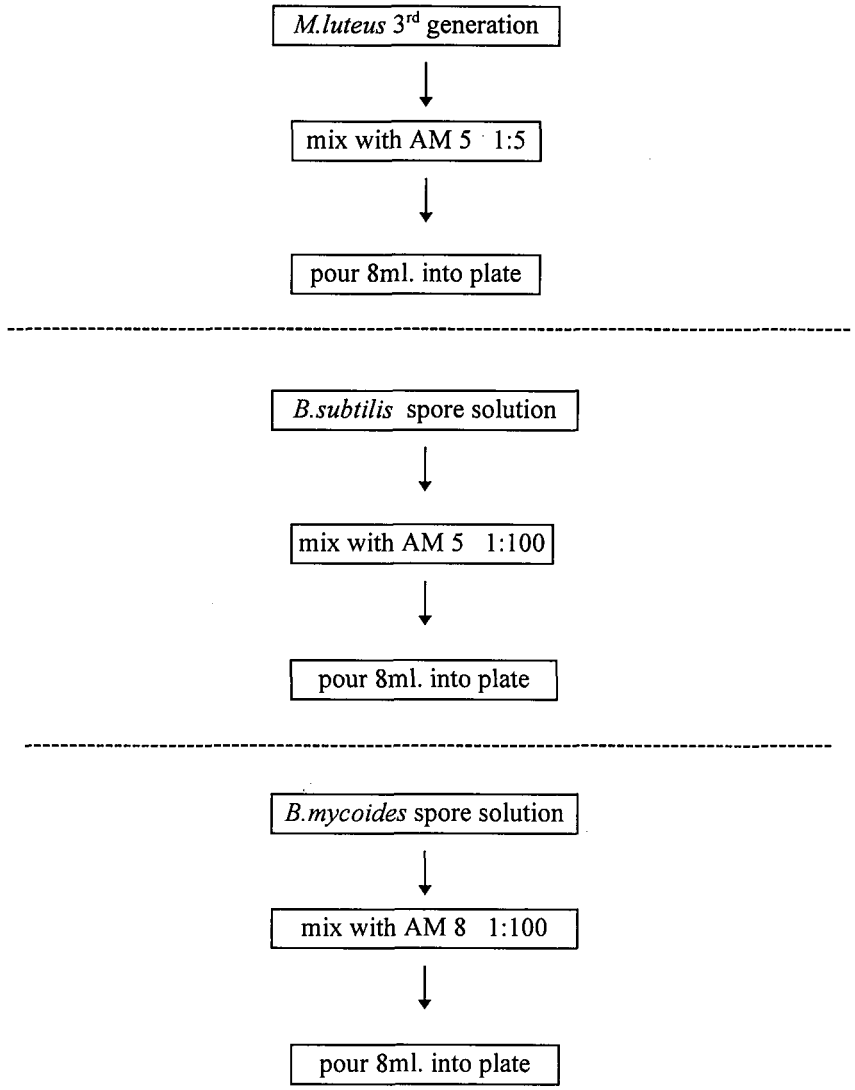
Preparation of Assay Strain Solution






B.cereus var.mycoides
inc. 35°C, 7 days.
streak on nutrient agar
scarp grown bacteria in saline solution tube
heat 65°C, 30 min. centrifuge 3000 rpm., 20 min.
put sediment into saline solution tube ➡ spore solution
make serial dilution 10^{-1},10^{-2},10^{-3}...
mix 1% of dilution with AM8 and pour 8ml into plate
put paper disc wetted with 0.25μg/ml OTC solution
inc. 30°C, 18 hr.
find out 14±1mm. inhibition zone diameter
(spore suspension contain 10^{7}-10^{8} spore/ml)

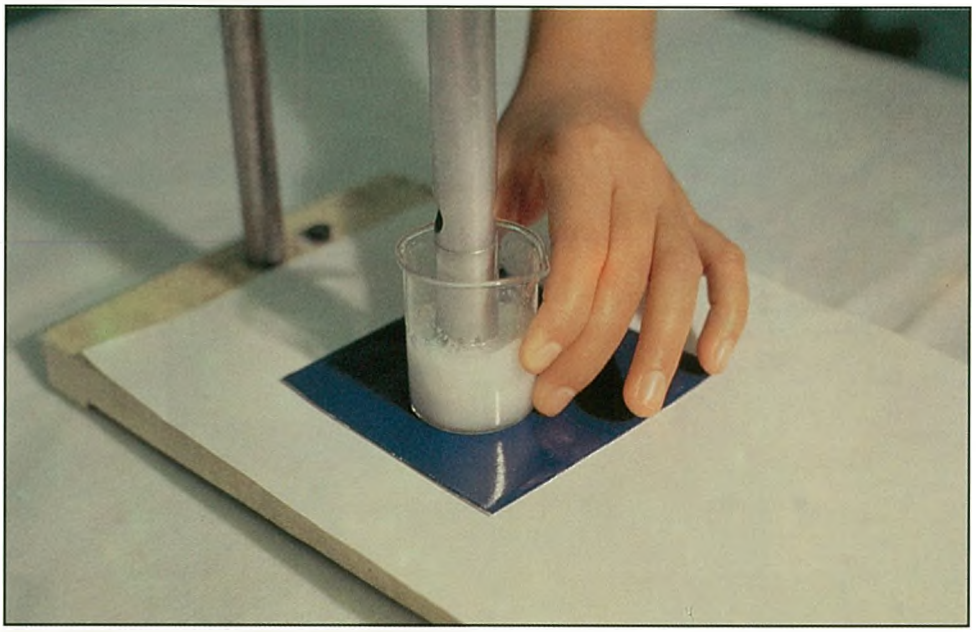
Preparation of Assay Plates



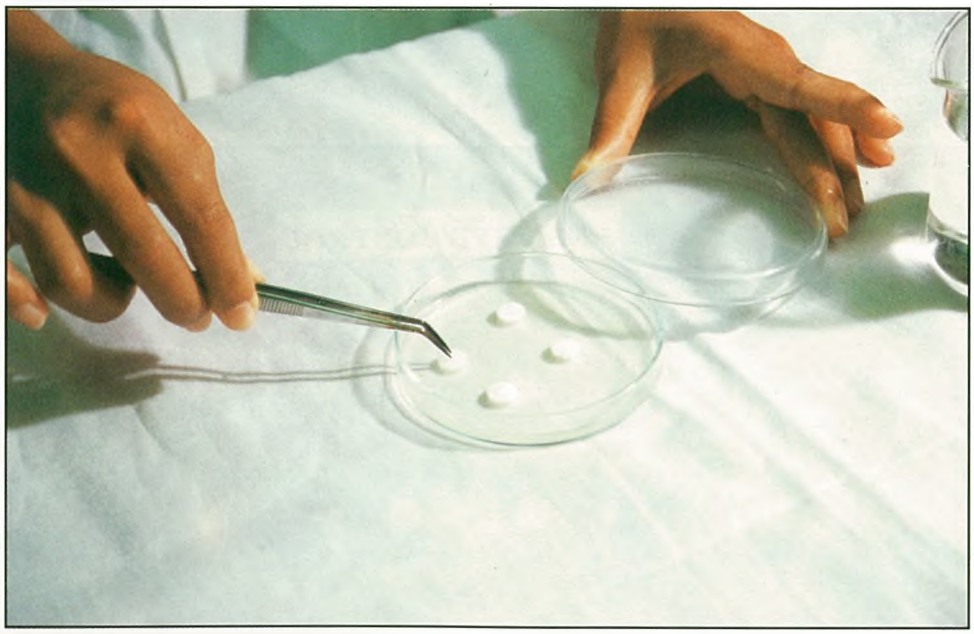
Determination of Oxytetracycline Residues by Microbiological Assay

Shrimp Sample

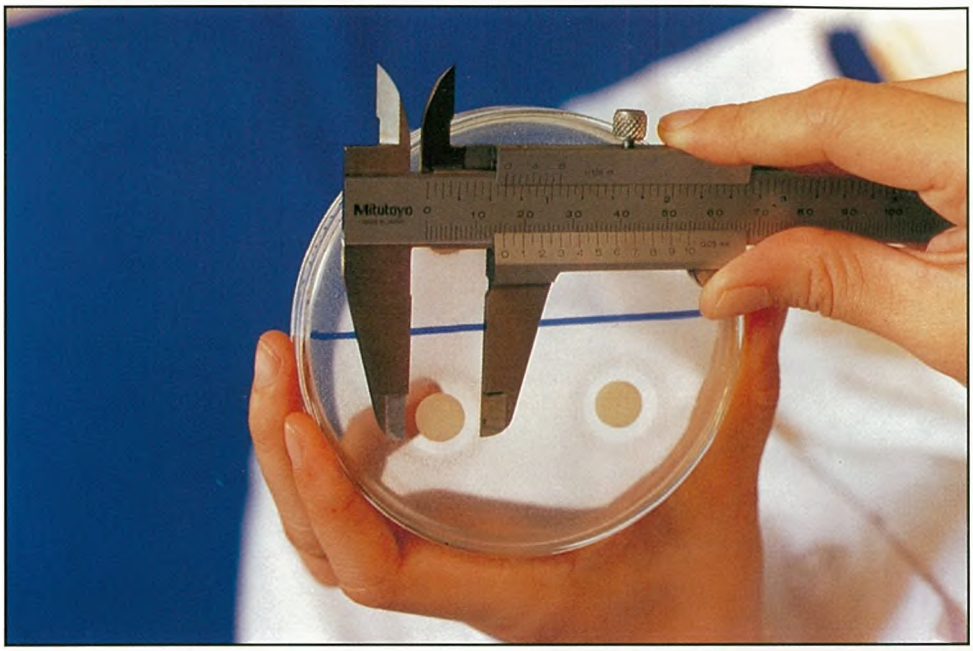
(1) Peeled and blend shrimp sample. → **(2) Weigh 5.0 g. of samples + 20ml citric acid-acetone buffer solution.**



(3) Homogenize at 5,000 rpm for 1 min. → (4) Filter through filter paper Whatman No.1



(5) Dip a paper disk into the sample solution. Put disk on an agar plate and → (6) use the paper disk wetted with citric acid-acetone buffer solution as a negative control. → (7) Incubate 30°C 18 hours.



(8) If diameter of inhibition zone is greater than 12 mm. the result is positive.

→

(9) Confirm that negative control of citric acid-acetone result is negative.

Positive sample, negative control